

Cell Lineage Determination and the Control of Neuronal Identity in the Neural Crest

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The diverse cell types of complex tissues such as the blood and the brain are generated from self-renewing, multipotent progenitors called stem cells (for reviews, see Hall and Watt 1989; Potten and Loeffler 1990; Morrison et al. 1997). These stem cells must generate progeny of different phenotypes, in the correct proportions, sequence, and location. The manner in which this is accomplished is not well understood. It is clear that the local microenvironment of stem cells has an important influence on their development, as do transcription factors that act within the cells. However, the manner in which such signals and transcription factors interact to control lineage determination by multipotent stem cells is poorly understood. To address this issue, it is necessary to both alter the expression of transcription factors in stem cells and challenge the cells by altering their environment to determine their state of lineage commitment. There are relatively few experimental systems in which such combined genetic and cell biological manipulations of stem cells are feasible.

FATE AND POTENTIAL OF NEURAL CREST CELLS IN AVIAN EMBRYOS

We have studied the control of lineage commitment by stem cells in the neural crest. The neural crest is a migratory population of progenitor cells that detaches from the dorsolateral margins of the neural tube and migrates to distant locations throughout the embryo (Fig. 1). Fate-mapping experiments in amphibian and avian embryos have demonstrated that the crest generates a diverse array of neural and mesectodermal derivatives (for reviews, see Le Douarin 1982; Bronner-Fraser 1993a). These derivatives include the neurons and glia of the peripheral nervous system, melanocytes, smooth muscle cells of the cardiac outflow tracts, and the bones and cartilage of the face. Some crest derivatives are generated only at certain positions along the anteroposterior axis (A/P) (Le

Douarin 1980). However, the crest also generates diverse derivatives at a single axial level. For example, neural crest cells in the thoracolumbar region of the trunk generate sensory neurons, sympathetic neurons, Schwann cells, adrenal chromaffin cells, and melanocytes (Le Douarin 1986).

Interspecific grafting experiments in avian embryos have revealed that the developmental potential of the crest is relatively homogeneous along the A/P axis, with the exception of craniofacial mesenchyme which apparently cannot be generated by transplanted trunk neural crest (Le Douarin 1982). This implies that the fate of neural crest cells is controlled by environmental signals. Such signals could act before, during, or after migration from the dorsal neural tube (Bronner-Fraser 1992). A major problem in neural crest cell biology, therefore, is to identify such environmental signals and their sources and to understand their mechanism of action on the crest (for reviews, see Stemple and Anderson 1993; Wehrlehaber and Weston 1997).

MULTIPOTENCY AND DEVELOPMENTAL RESTRICTION OF NEURAL CREST CELLS

The pluripotency of the crest revealed by transplantation experiments could reflect a homogeneous population of pluripotent cells, or a mixture of committed cells. In vivo lineage tracing experiments in chick have shown that many premigratory crest cells are multipotent (Bronner-Fraser and Fraser 1988). This observation is consistent with the results of in vitro clonal analysis of quail neural crest cells (Sieber-Blum and Cohen 1980; Baroffio, et al. 1988). The fact that the crest contains multipotent cells, and that the fate of these cells is influenced by the embryonic environment, leads to two extreme models for how lineage commitment is accomplished. On the one hand, specific environmental signals may instruct uncommitted cells to choose one fate at the expense of others. Such a mechanism would be termed "instructive." On the other hand, uncommitted cells may undergo lineage restriction by a cell-autonomous mechanism (either stochastic or deterministic), and environmental factors may permit the survival and proliferation of appropriate cells in the appropriate place. Such a mechanism would be termed "selective" (Morrison et al. 1997). One of our

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Neural Crest Derivatives

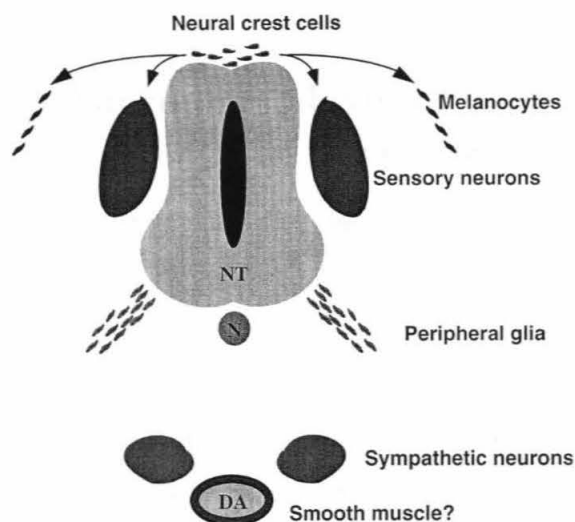


Figure 1. Cell types produced by multipotential stem cells in the trunk neural crest. The contribution to the vascular smooth muscle of the dorsal aorta is speculative. (Reprinted, with permission, from Anderson 1997.)

major objectives has been to determine which of these mechanisms is operative in the neural crest.

It is believed that the neural crest, like the hematopoietic system, undergoes progressive restrictions in the developmental potentials of individual progenitor cells (for discussions, see Anderson 1989; Sieber-Blum 1990; Le Douarin et al. 1991; Weston 1991). Subpopulations of crest-derived cells with apparently restricted developmental capacities have been detected in avian peripheral ganglia and other tissues colonized by crest cells (Sieber-Blum et al. 1993). However, rigorously documenting such restrictions requires the ability to challenge individual crest cells by exposing them to novel environments or to defined molecules capable of instructing alternative fates. With few exceptions (Sieber-Blum 1991; Lo and Anderson 1995) this has not been achieved: Either populations of crest cells have been challenged by transplantation *in vivo* (Weston and Butler 1966; Le Douarin 1986; Artinger and Bronner-Fraser 1992) or individual crest cells have been analyzed *in vitro* but not challenged with different instructive signals to assess their state of commitment (Duff et al. 1991; Sextier-Sainte-Claire Deville et al. 1992, 1994). The nature of the restricted sublineages that arise from the neural crest therefore remains poorly defined. Furthermore, the logic of how such restrictions emerge is not clear; various hierarchical, sequential, and stochastic models for lineage segregation have been put forth (for review, see Anderson 1993), and none have yet been experimentally validated.

We have approached the problem of neural crest cell lineage determination by isolating neural crest (Stemple and Anderson 1992) and crest-derived (Michelsohn and Anderson 1992; Lo and Anderson 1995) cells from embryos at different stages of development, and developing

in vitro clonogenic assay systems (Stemple and Anderson 1992) where the developmental capacities of the cells, and their responses to environmental signals, can be assessed. This has allowed the identification of specific signals that can influence crest cell fate, and a determination of whether they act selectively or instructively (Shah et al. 1994, 1996). We have also begun to identify transcription factors important in the specification of different crest sublineages (Johnson et al. 1990; Ma et al. 1996; Sommer et al. 1996). By combining these cell biological and molecular approaches, we have begun to dissect the interplay between environmental signals and cell-intrinsic determinants in the control of neural crest lineage diversification. Here we review some of the concepts and particulars that have emerged from this experimental approach.

SELF-RENEWAL OF MULTIPOTENT MAMMALIAN NEURAL CREST CELLS

A defining characteristic of stem cells in other systems, such as the hematopoietic system, is their ability to self-renew; i.e., to divide to produce progeny with the same developmental capacities (Hall and Watt 1989; Potten and Loeffler 1990; Morrison et al. 1997). Although neural crest cells in avian embryos have been demonstrated to be multipotent (for review, see Bronner-Fraser 1993b), their ability to self-renew has not been addressed. We isolated a population of rat neural crest cells using antibodies to the low-affinity nerve growth factor (NGF) receptor (p75^{L^{NTR}}) as a cell surface marker (Chandler Parsons et al. 1984) and have demonstrated that these cells, like their avian counterparts, are multipotent (Stemple and Anderson 1992). In clonal culture, they are able to generate at least three of the cell types that normally derive from the crest *in vivo*: autonomic neurons, glia, and smooth muscle. Moreover, by subcloning these cells, we have demonstrated that they are capable of self-renewal, at least for a limited number of generations (6–10) (Stemple and Anderson 1992). Thus, these neural crest cells exhibit multipotency and self-renewal, two properties of stem cells in the hematopoietic system (Davis and Reed 1996). More recently, similar studies have been performed for multipotent neural progenitors from the brain (Davis and Temple 1994; Gritti et al. 1996; Johe et al. 1996), supporting the idea that neural stem cells exist in the central nervous system (CNS) as well as in the peripheral nervous system (PNS).

The demonstration of self-renewal by multipotent neural progenitors *in vitro* raises the question of whether such self-renewal also occurs *in vivo*, and if so for how long. Multipotent neural progenitors have been recovered from the adult mammalian brain (Reynolds and Weiss 1992; Lois and Alvarez-Buylla 1993; Palmer et al. 1997; for review, see Aubert et al. 1995). Whether these adult stem cells reflect the self-renewal of embryonic stem cells, the persistence of such cells in a quiescent state, or rather *de novo* generation from a pre-stem cell remains to be determined. The existence of stem cells in adult neural-crest-derived structures has not yet been demonstrated. How-

ever, in avian embryos, multipotent cells can be recovered from some tissues colonized by the neural crest at late developmental stages (Sieber-Blum et al. 1993). These data are consistent with the idea that neural crest cells may undergo some self-renewal in vivo as well as in vitro.

IDENTIFICATION OF INSTRUCTIVE SIGNALS THAT INFLUENCE LINEAGE COMMITMENT BY NEURAL CREST STEM CELLS IN VITRO

We have identified several growth factors that can promote the differentiation of neural crest cells to specific lineages. Glial growth factor-2 (GGF2), a neuregulin (Marchionni et al. 1993; for review, see Burden and Yarden 1997), promotes the differentiation of peripheral glial (Schwann) cells (Shah et al. 1994). Members of the transforming growth factor- β (TGF- β) superfamily, TGF β 1-3 and BMP2/4, promote the differentiation of smooth muscle cells and autonomic neurons, respectively (although the BMPs also produce some smooth muscle cells in addition to neurons) (Fig. 2) (Shah et al. 1996). Clonal analysis and sequential observations of single clones have excluded the possibility that these factors act to promote the selective survival of a subset of founder cells pre-committed to a particular fate or that they selectively kill cells within clones that commit to the "wrong" fates (Shah et al. 1994, 1996). Thus, we have concluded that these factors act instructively rather than selectively.

The neural crest represents the first case in which growth factors have been shown to influence lineage determination by multipotent stem cells in an instructive rather than a selective manner. Similar data have recently been obtained for stem cells from the brain (Johe et al. 1996). In contrast, the available data in the hematopoietic system support selective rather than instructive actions for growth factors on lineage commitment (Fairbairn et al. 1993). Whether this reflects a fundamental difference in the way that the two systems utilize growth factors to control lineage decisions, or rather that instructive factors for hematopoietic stem cells have simply not yet been identified, remains to be determined.

We should point out that the developing nervous system also employs selective mechanisms in its development. For example, the neurotrophins (e.g., NGF, BDNF, and NT-3) act to promote the survival of subsets of peripheral sensory and autonomic neurons (for review, see

Thoenen 1991). Indeed, even GGF/neuregulin has been shown to promote the survival of lineage-committed Schwann cell progenitors (Dong et al. 1995), as well as the proliferation of mature Schwann cells (Lemke and Brookes 1984). Thus, the same factor may initially act as an instructive fate-determination signal and later as a selective survival factor or mitogen, at successive stages in the development of a particular lineage (Topilko et al. 1997).

Neural crest cells are likely to encounter multiple signals in their local environment in vivo. It is therefore important to understand how the cells integrate such opposing influences. Both GGF2 and TGF- β are able to antagonize the neurogenic influences of BMP2 on NC-SCs (Shah and Anderson 1997). However, the cells display very different sensitivities to these antagonistic interactions: TGF- β antagonizes BMP2 at saturating concentrations of the latter, whereas antagonism by GGF2 is only detectable at BMP2 concentrations 50–100-fold below saturation. These differences do not simply reflect different dosage sensitivities to GGF and TGF- β , they also reflect the fact that commitment to a glial fate in GGF2 occurs much more slowly (48–96 hours) than it does to a smooth muscle fate in TGF- β (≤ 24 hours) (N.M. Shah and D.J. Anderson, in prep.). The reasons for these differences in the kinetics of commitment are not yet clear.

The antagonistic interactions between TGF- β and BMP2 may reflect competition for limiting quantities of a shared signaling component, such as DPC4/Smad4 (A. Candia et al., pers. comm.). The antagonism between BMP2 and GGF2 is also interesting in light of recent data identifying antagonistic interactions between *Drosophila* homologs of these factors, DPP and D-EGF, in tracheal morphogenesis (see Shilo et al., this volume) as well as in follicle cell fate determination during oogenesis (L. Dobens and L. Raftery, pers. comm.). The molecular basis of this antagonism remains to be elucidated.

ROLE OF IDENTIFIED INSTRUCTIVE SIGNALS IN CONTROLLING NEURAL CREST DEVELOPMENT IN VIVO

The identification of GGF, TGF- β s 1-3, and BMP2/4 as instructive signals for neural crest lineage determination in vitro immediately raises several new questions. Do these growth factors play a role in determining neural crest cell fate in vivo, and if so what cells produce them and what regulates their production? How do these extracellular signals interact with transcriptional regulators to cause cells to commit to particular fates?

All of the identified factors are expressed in vivo at appropriate places and times to influence neural crest cell fate in a manner suggested by their actions in vitro. For example, several TGF- β s are found in the developing outflow tracts of the heart, where neural crest cells contribute to smooth muscle (for review, see Kingsley 1994). Knockouts in some of these growth factors lead to cardiac defects (for review, see Moses and Serra 1996), although whether these defects specifically affect crest-derived

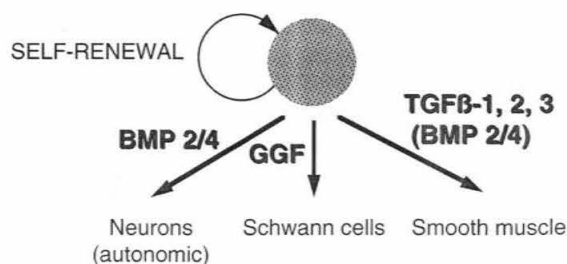


Figure 2. Instructive growth factors controlling lineage determination by neural crest stem cells. (Reprinted, with permission, from Shah et al. 1996 [copyright Cell Press].)

smooth muscle is not yet known. BMP2 and BMP4 are found in locations near or in which autonomic neurons develop: For example, the former is present in the dorsal aorta (Reissman et al. 1996; Shah et al. 1996) (Fig. 3A), the site of sympathetic gangliogenesis, whereas the latter is present in the gut (Bitgood and McMahon 1995; Lyons et al. 1995), the site of enteric neurogenesis. Unfortunately, knockouts in these genes die at a stage of em-

bryogenesis too early to assess their requirement in the development of these neural-crest-derived autonomic ganglia (Winnier et al. 1995; Zhang and Bradley 1996; for review, see Hogan 1996). Therefore, tissue-specific knockouts of these factors or their receptors (Mishina et al. 1995) will be required to address their requirement for autonomic neurogenesis *in vivo*.

GGF/neuregulins are expressed by motor, sensory, and sympathetic neurons, near which peripheral glia develop (Marchionni et al. 1993; Meyer and Birchmeier 1994). We have suggested that this neuronal expression may constitute part of a negative feedback loop whereby neurons signal neighboring uncommitted stem cells to generate glia (Shah et al. 1994). The neuregulin knockout contains a reduced number of Schwann cells associated with peripheral nerve (Meyer and Birchmeier 1995), consistent with the *in vitro* actions of GGF2. However, whether the mutant phenotype reflects an *in vivo* action of the factor in lineage determination, progenitor cell survival (Dong et al. 1995), differentiation (Murphy et al. 1996), or proliferation (Lemke and Brookes 1984) is not yet known.

CONTROL OF AUTONOMIC NEURONAL LINEAGE DETERMINATION: INTERACTIONS BETWEEN BMP2/4 AND MASH1

How extracellular signals and transcription factors interact to control lineage commitment is beginning to emerge from studies of autonomic neurogenesis. Mammalian achaete-scute homolog-1 (MASH1) is a basic helix-loop-helix (bHLH) transcription factor that is specifically expressed in precursors of autonomic (but not sensory) neurons (Fig. 3C) (Lo et al. 1991). MASH1 is essential for the development of these neurons as shown by targeted mutagenesis in mice (Guillemot et al. 1993; Blaugrund et al. 1996). The fact that BMP2 and BMP4 promote expression of autonomic phenotypes *in vitro* (Varley et al. 1995; Reissman et al. 1996; Shah et al. 1996; Varley and Maxwell 1996) raised the question of whether these factors are also involved in the regulation of expression of MASH1. The available evidence suggests that they are. Purified recombinant BMP2 induces MASH1 expression in cultured neural crest cells within 6–12 hours (Fig. 4) (Shah et al. 1996) (a time by which many cells have not yet divided), suggesting that the growth factor acts directly on the cells to promote expression of the transcription factor. As mentioned earlier, BMP2 mRNA is expressed in the wall of the dorsal aorta (Fig. 3A), and this expression is detected at the time that MASH1⁺ precursors of sympathetic autonomic neurons can first be observed adjacent to this structure (Fig. 3B,C) (Shah et al. 1996). Furthermore, explants of dorsal aorta tissue can induce MASH1 expression in cultured neural crest cells in a manner sensitive to inhibition by noggin (A. Groves and D.J. Anderson, unpubl.). These data suggest that neural crest cells begin to express MASH1 when they migrate to peripheral tissues that are local sources of BMP2 or BMP4.

What are the consequences of MASH1 expression for

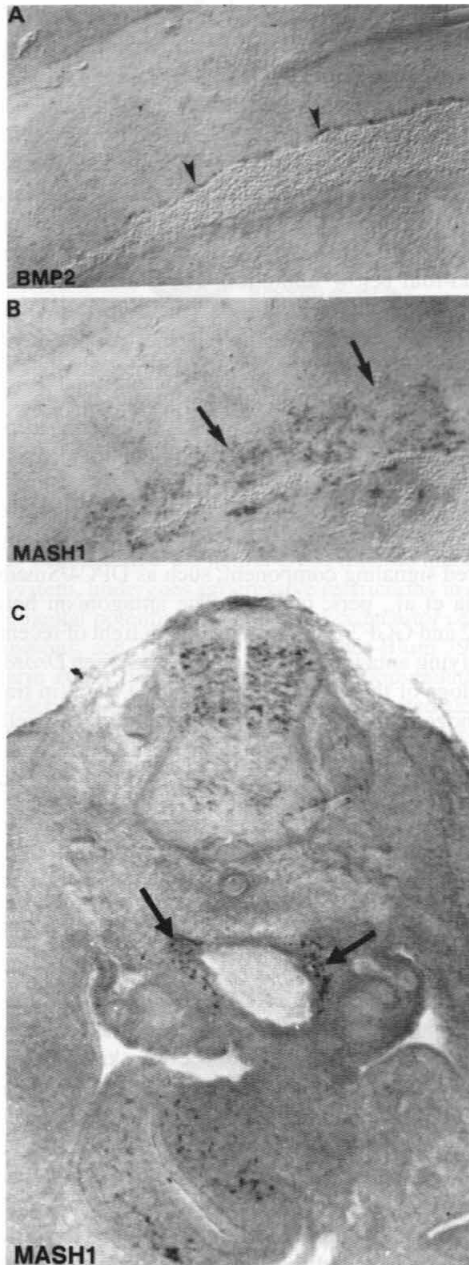


Figure 3. Coincident expression of BMP2 mRNA in the wall of the dorsal aorta (A, arrowheads) at the time that neural crest cells expressing MASH1 mRNA (B, arrows) or protein (C, arrows) can be detected adjacent to this structure. A and B are adjacent sagittal sections, whereas C is a transverse section. MASH1⁺ cells are also detected ventral to the sympathetic chain in the gut (C), which is known to contain BMP4 (Bitgood and McMahon 1995).

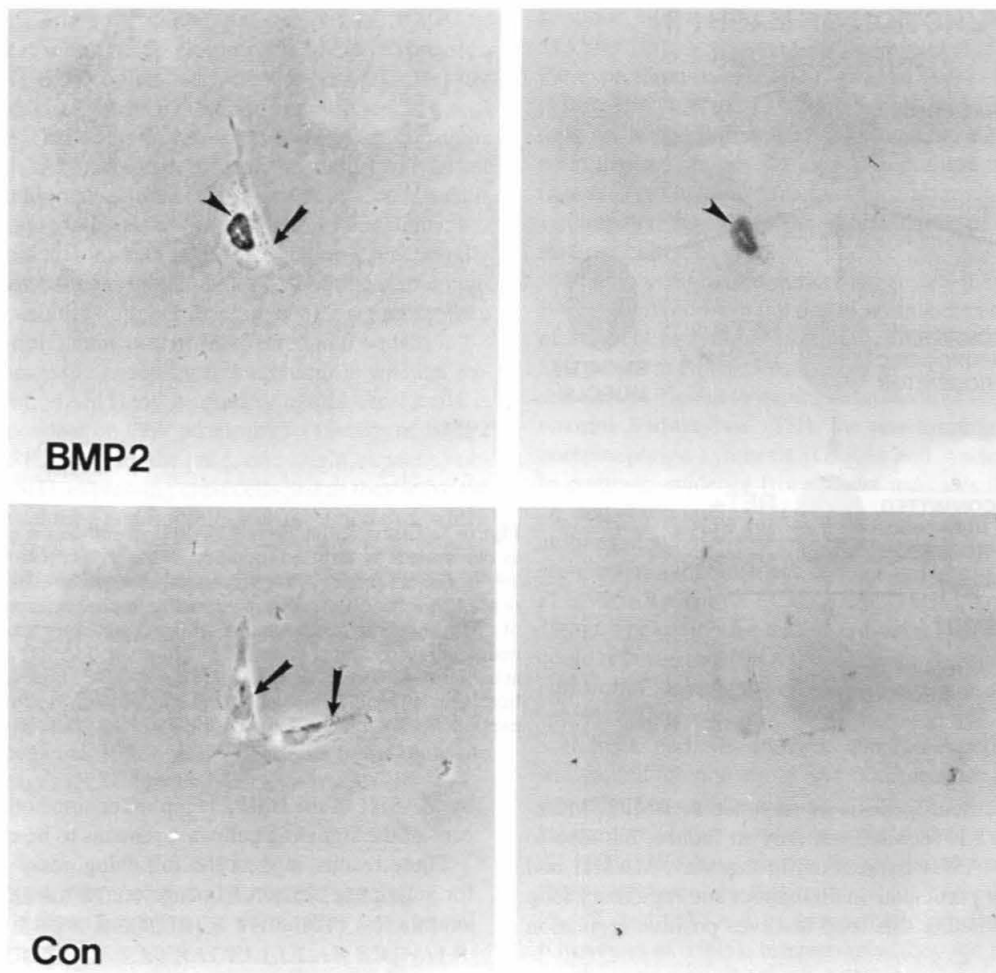


Figure 4. Induction of MASH1 protein in isolated neural crest stem cells by BMP2 in vitro. Arrowhead (upper panels) indicates the nucleus of a single MASH1-immunoreactive neural crest cell 12 hr after exposure to 50 ng/ml BMP2.

the fate of neural crest cells? Detailed analysis of *Mash1* mutant embryos has suggested that the gene is essential for the execution of terminal neuronal differentiation and is required only after the cells have already begun to express some neuronal genes (such as neurofilament 150-kD subunit) and have likely committed to a neuronal fate (Fig. 5) (Sommer et al. 1995). However, MASH1 expression is first detected prior to expression of such early neuronal markers (Fig. 4) (Lo et al. 1991; Sommer et al. 1995; Shah et al. 1996), raising the possibility that it has an earlier function in autonomic neurogenic lineage commitment not revealed by the null mutation.

To address this possibility, we have examined the relationship between expression of MASH1 and the commitment of neural-crest-derived cells to an autonomic neuronal fate. Postmigratory neural crest cells that are progenitors of autonomic enteric neurons can be isolated from fetal (E12.5–E14.5) rat gut using the receptor tyrosine kinase c-RET (Pachnis et al. 1993) as a cell surface marker (Lo and Anderson 1995). The majority (>85%) of such cells already express MASH1. Clonal analysis of these isolated c-RET⁺ cells revealed that many of the cells (25–50%) are already committed to a neuronal fate

(Lo and Anderson 1995). However, $\geq 50\%$ of the cells are not committed to a neuronal fate, but rather generate non-neuronal derivatives, including glia and smooth muscle (Lo et al. 1997). These data suggest that, qualitatively at least, expression of MASH1 cannot be sufficient for commitment to a neuronal fate (although it may depend on the quantitative level of MASH1 expression, a parameter that has not yet been examined).

Does MASH1 have any function in the uncommitted postmigratory crest cells? A gain-of-function experiment suggests that MASH1 is required to maintain competence for neuronal differentiation in these cells. Virtually all c-RET⁺ cells are initially competent for neurogenesis, since those cells which have not yet committed to a neuronal fate can be converted to neurons by BMP2 (Lo et al. 1997). This neurogenic competence is, however, lost with time in culture. In parallel, there is a loss of endogenous MASH1 expression, which can be prevented by maintaining the cells in BMP2. Constitutive expression of MASH1 from a retroviral vector, in turn, maintains competence for neurogenesis induced by BMP2 (Lo et al. 1997). These data suggest that expression of MASH1 in c-RET⁺ postmigratory neural crest cells maintains com-

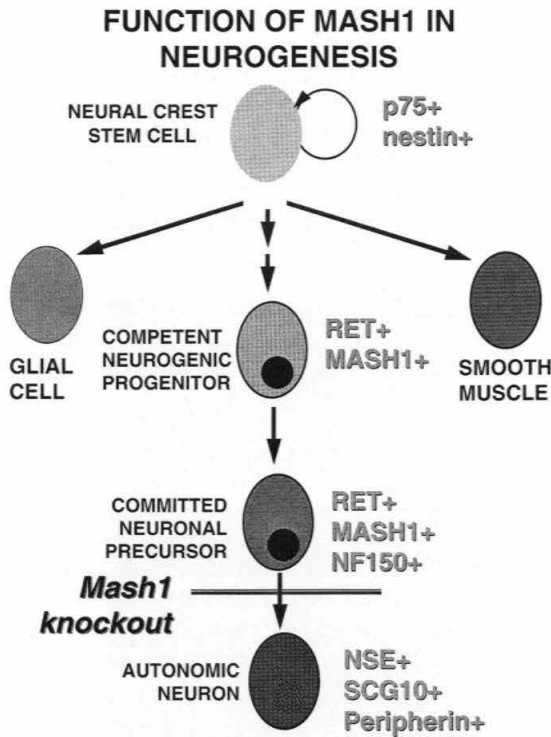


Figure 5. Essential function of MASH1 in autonomic neurogenesis as determined by targeted mutation in the mouse (Guillemot et al. 1993). The absence of *Mash1* function causes an arrest of autonomic neurogenesis at a stage when precursor cells already express a subset of neuron-specific genes (eg., NF150) and are likely committed to a neuronal fate (Sommer et al. 1995). However, MASH1 is expressed at earlier stages in the lineage, where it may have a nonessential function in maintaining competence for neurogenesis, as shown by over-expression experiments (Lo et al. 1997).

petence for neurogenesis in response to BMP2. Moreover, BMP2 is required not only to induce, but also to maintain MASH1 expression. In this way, MASH1 and BMP2 may participate in an indirect autoregulatory loop (Fig. 6). Whether this loop involves positive regulation

by MASH1 of the BMP2 receptor, or some other component of the signaling pathway, remains to be explored.

These results suggest the following possible scenario for autonomic neuronal lineage commitment. Migrating neural crest cells arrive at peripheral target tissues (e.g.,

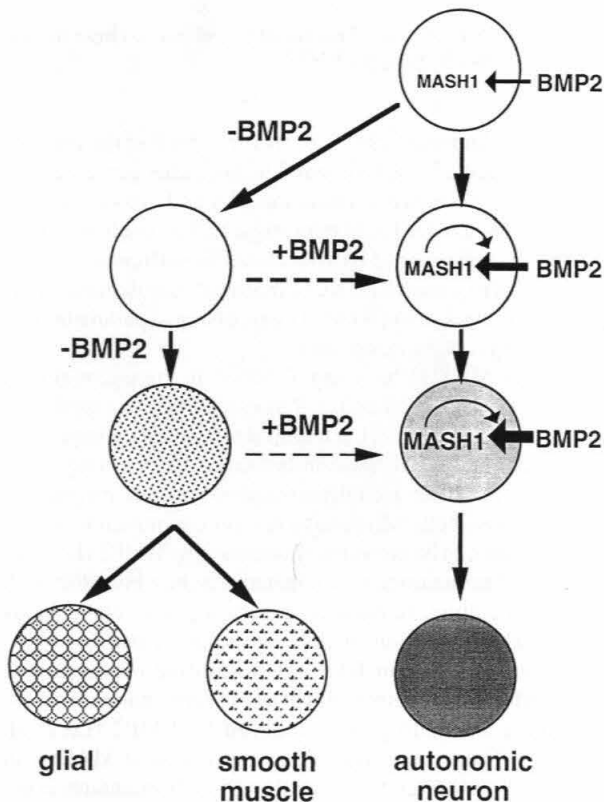


Figure 6. MASH1 participates in a positive autoregulatory loop with BMP2 that functions in maintaining competence for autonomic neurogenesis. The figure summarizes data obtained from analysis of MASH1 expression and function in a population of postmigratory neural crest cells isolated using monoclonal antibodies to c-RET (Lo and Anderson 1995). Maintenance of MASH1 expression in these cells in vitro requires continued exposure to BMP2; in turn, continued expression of MASH1 is required to maintain BMP2-responsiveness (Lo et al. 1997). If the cells are cultured in the absence of BMP2, expression of MASH1 is lost and the cells eventually irreversibly lose neurogenic capacity. (Reprinted, with permission, from Lo et al. 1997.)

dorsal aorta and gut), which produce a signal (BMP2 or BMP4) that induces expression of MASH1. Expression of MASH1 then confers competence to undergo further BMP2-mediated events that lead to commitment to a neuronal fate. This scenario raises several further questions that remain to be explored. What is the molecular basis of commitment to a neuronal fate? Does it involve a quantitative increase in levels of MASH1 expression, induction of cofactors that interact with MASH1, or a functionally distinct downstream factor? Does commitment to a neuronal fate require a higher level of BMP2 signaling than does the initial induction of MASH1, and if so why?

A further issue raised by this scenario is whether expression of MASH1 by migrating neural crest cells is solely dependent on their proximity to sources of BMP2 or BMP4. If that were the case, one might expect to observe MASH1-expressing crest cells just as they leave the dorsal neural tube and migrate near the ectoderm, which at early stages is a source of BMP4 (Liem et al. 1995). The fact that such cells are not observed *in vivo* suggests either that neural crest cells are initially not competent to express MASH1 in response to BMP2 or BMP4 when they first leave the dorsal neural tube or that the crest cells are protected from the effects of local BMP4 signaling by antagonists such as noggin (Smith and Harland 1992; Zimmerman et al. 1996), which is present in the roofplate at these stages (R.M. Harland, pers. comm.).

EXPRESSION OF A SYMPATHETIC PHENOTYPE REQUIRES THE INTEGRATION OF MULTIPLE EXTRACELLULAR SIGNALS

MASH1 is expressed in, and required for, the development of multiple autonomic neuronal subtypes. These subtypes can be distinguished by the kind of neurotransmitter they express. For example, sympathetic neurons synthesize norepinephrine, whereas parasympathetic neurons synthesize acetylcholine. Expression of MASH1 is not sufficient to specify these different autonomic subtypes, although it is necessary for this process (Guillemot et al. 1993). What other transcription factors are necessary to specify these different autonomic subtypes, and how is their expression controlled by extracellular signals?

Several transcription factors are specifically expressed in developing sympathetic ganglia besides MASH1, including Phox2a (Valarché et al. 1993), eHAND/Thg-1/Hxt (Cross et al. 1995; Cserjesi et al. 1995; Hollenberg et al. 1995), dHAND (Srivastava et al. 1995), and GATA-2 and GATA-3 (George et al. 1994; Tsai et al. 1994; Groves et al. 1995). Phox2a is a paired homeodomain protein; eHAND and dHAND are bHLH proteins; and GATA-2 and -3 are zinc finger proteins (which also function in the hematopoietic system [Briegleb et al. 1993; Tsai et al. 1994]). Phox2a, eHAND, and dHAND are, like MASH1, expressed by all autonomic sublineages. GATA-2 and GATA-3, in contrast, are expressed by sympathetic neurons but not by enteric or parasympathetic neurons (Fig. 7) (Groves et al. 1995). These tran-

scription factors appear to be expressed subsequent to MASH1 (Ernsberger et al. 1995; Groves et al. 1995), and the expression of eHAND (Ma et al. 1997) and Phox2a (M.-R. Hirsch et al.; L. Lo et al.; both in prep.) is dependent on MASH1 function. Although their functions in neurogenesis are not yet established, these transcription factors serve as useful markers to analyze the role of environmental signals in the specification of the sympathetic phenotype.

Evidence discussed earlier suggested that BMP2 (or BMP4) derived from the dorsal aorta induces expression of MASH1 in sympathetic precursors. However, in NCSCs grown at clonal density, neither BMP2 nor the dorsal aorta are sufficient to induce expression of GATA-2/-3 or tyrosine hydroxylase (TH), the rate-limiting enzyme in norepinephrine synthesis (Groves and Anderson 1996). In contrast, evidence from others indicates that BMP-2, -4, and -7 can induce TH expression in high-density mass cultures of avian neural crest cells, which, unlike NCSCs, are grown in the presence of fetal calf serum (Varley et al. 1995; Reissman et al. 1996; Varley and Maxwell 1996). The reason for this discrepancy is not clear, but it could reflect a requirement for density-dependent signals, additional signals provided by fetal calf serum, or a species difference.

In avian embryos, surgical ablation experiments have shown that the notochord and floorplate are required for the induction of catecholamine histofluorescence in neural crest cells aggregating near the dorsal aorta (Stern et al. 1991). We have shown by similar experiments that these structures are also required for expression of TH, Phox2, and GATA-2 by sympathetic precursors *in vivo* (Groves et al. 1995). Interestingly, they are not required for the induction of either MASH1 (the avian homolog of MASH1; Jasoni et al. 1994) or SCG10, a pan-neuronal marker (Stein et al. 1988). These observations are consistent with the finding that in cultured mammalian NCSCs, BMP2 leads to induction of MASH1, SCG10, and a neuronal morphology, but not to expression of TH or GATA-2 (Groves and Anderson 1996). (Although, as mentioned above, in the avian system, BMPs alone appear sufficient to induce expression of TH [Varley et al. 1995; Reissman et al. 1996; Varley and Maxwell 1996]; in most cases, these TH⁺ cells do not coexpress neuronal markers [Christie et al. 1987].)

Taken together, these data suggest that the expression of pan-neuronal components of the sympathetic phenotype can be experimentally uncoupled from the expression of subtype-specific components, such as neurotransmitter synthesis. Expression of these different components may therefore be under the control of different genetic subprograms, which in turn may be under distinct environmental control. BMP2-like signals from the dorsal aorta and gut induce MASH1 and a subprogram leading to expression of pan-neuronal and some pan-autonomic properties, whereas additional signals from the notochord and floorplate may be required for expression of a subprogram leading to expression of the sympathetic neurotransmitter phenotype (Fig. 7). These subprograms are not completely independent, however, as MASH1 is required for expression of both the pan-neuronal and sub-

COMBINATORIAL CONTROL OF AUTONOMIC DEVELOPMENT BY TRANSCRIPTION FACTORS

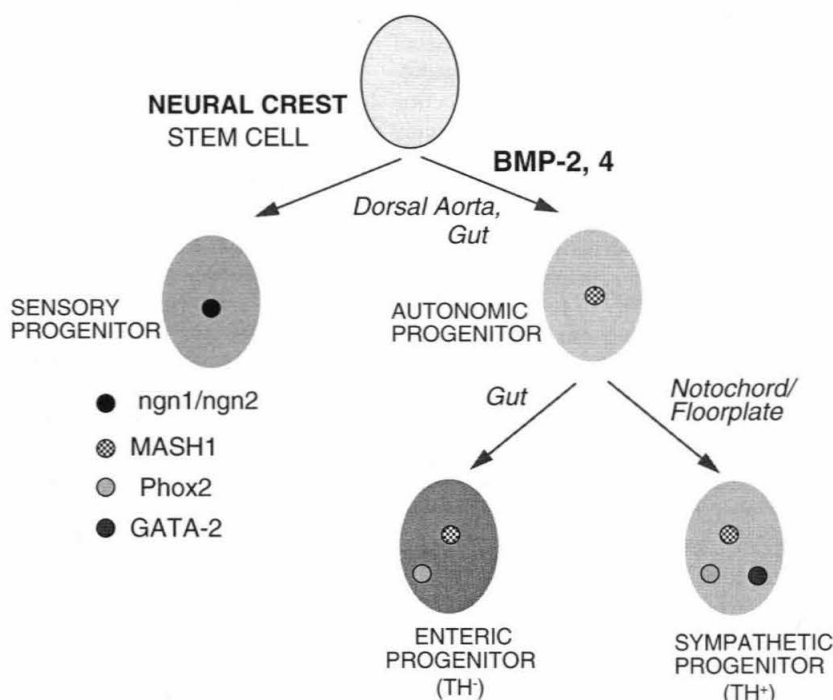


Figure 7. Different combinations of specifically expressed transcription factors are correlated with different autonomic neuronal identities. Progenitors in all three major autonomic sublineages (sympathetic, parasympathetic, and enteric) express MASH1, which is likely induced by BMP2 or BMP4 present in tissues such as the dorsal aorta and gut. These progenitors are likely exposed to additional signals in a location-specific manner, which cause the expression of sublineage-specific transcription factors such as GATA-2. Phox2 is also expressed by most or all autonomic sublineages, and it appears to be a downstream target of MASH1 (L.-C. Lo et al., in prep.).

type-specific components of the sympathetic phenotype (Guillemot et al. 1993; Sommer et al. 1995).

SPECIFICATION OF DIFFERENT SENSORY NEURON SUBTYPES MAY INVOLVE THE ACTION OF DISTINCT BUT RELATED bHLH PROTEINS

Although significant progress has been made in understanding the specification of autonomic lineages, much less is known about the specification of sensory lineages. The segregation of the sensory and autonomic lineages represents a major divergence point in the development of the neural crest (Le Douarin 1986), comparable to the segregation of lymphoid and myelo-erythroid lineages in hematopoiesis (Ikuta et al. 1992; Morrison et al. 1994). The logic and mechanisms underlying this lineage segregation event are not understood. Moreover, like the autonomic lineage, the sensory lineage comprises multiple neuronal subtypes, such as those mediating touch, pain, and posture (Snider 1994). In contrast to different subtypes of autonomic neurons, which develop in distinct embryonic locations, different subclasses of sensory neurons develop in a common location, the dorsal root gan-

glia, adjacent to the spinal cord. The development of sensory neurons thus poses a different set of problems than does that of autonomic neurons.

Recently, we identified a subfamily of related bHLH proteins, the neurogenins, that are likely to have a key role in the development of the sensory lineage (Ma et al. 1996; Sommer et al. 1996). Neurogenins (ngns)-1, -2, and -3 (also known as MATH4C, A and B, respectively; Gradwohl et al. 1996; Cau et al. 1997) are closely related to NeuroD and its relatives (for review, see Lee 1997), but form a distinct subfamily (Sommer et al. 1996). In vivo, *ngn1* and *ngn2* are expressed in developing sensory but not autonomic ganglia, in a manner complementary to MASH1 (Ma et al. 1996; Sommer et al. 1996). Interestingly, this complementarity extends to the central nervous system as well (Ma et al. 1997), suggesting that MASH1 and the *ngns* have a broader role in the specification of different neuronal subtypes. (An exception is the olfactory placode, where MASH1, *ngn1*, and NeuroD have been shown to function in a cascade that likely acts within the same lineage [Cau et al. 1997].)

The function of neurogenins has been investigated by ectopic expression experiments in *Xenopus*. Injection of mRNA encoding *Xenopus* neurogenin-related-1 (Xngnr-

1) causes a massive induction of ectopic neurogenesis, both within the neural tube and in the flanking nonneurogenic ectoderm (Ma et al. 1996), similar to the phenotype caused by ectopic expression of XNeuroD (Lee et al. 1995). Ectopic expression of Xngnr-1 also induces ectopic expression of endogenous XNeuroD (Fig. 8) (Ma et al. 1996). In contrast, injection of XNeuroD mRNA fails to induce expression of endogenous Xngnr-1. These gain-of-function data suggest that Xngnr-1 can function as a neuronal determination gene and that it acts upstream of endogenous XNeuroD in a unidirectional cascade controlling neurogenesis (Ma et al. 1996). The early expression of endogenous Xngnr-1 and its interaction with the lateral inhibition machinery mediated by XNotch-1 and XDelta-1 (Fig. 8) (Chitnis et al. 1995; Chitnis and Kintner 1996; Ma et al. 1996) are consistent with the idea that Xngnr-1 normally does function in the process of neuronal determination, *in vivo*.

The function of the neurogenins in higher vertebrates is not yet established. However, the expression patterns of the genes are of interest in relation to the issue of sensory neuron subtype specification. In particular, *ngn1* and *ngn2* are expressed in mostly complementary subsets of cranial sensory ganglia: *ngn1* is most highly expressed in "proximal" ganglia whose neurons derive from the cranial neural crest and/or the otic and trigeminal placodes,

whereas *ngn2* is prominently expressed in "distal" ganglia whose neurons derive from the epibranchial placodes (Gradwohl et al. 1996; Sommer et al. 1996). These data indicate a correlation between the expression of *ngns* and different sensory neuron subtypes. In trunk dorsal root sensory ganglia, both *ngn1* and *ngn2* are expressed; however, they are expressed sequentially (*ngn2* followed by *ngn1*) rather than simultaneously (Sommer et al. 1996). The expression of these two genes in distinct subsets of cranial sensory ganglia suggests, by analogy, that in the DRG, these same genes could specify distinct subtypes of trunk sensory neurons (Snider 1994). Targeted mutations in the *ngns* are in progress to examine this possibility.

SUMMARY

The molecular mechanisms underlying the determination of neuronal identity in the vertebrate peripheral nervous system are only just beginning to come into focus. Many of these mechanisms, such as the involvement of cascades of bHLH transcription factors and lateral inhibition via the Notch-Delta system, appear to have been conserved from *Drosophila* (Ghysen et al. 1993; Jan and Jan 1993). The way in which these genetic circuits are controlled by instructive growth factors, and the manner in which they lead to expression of a particular neuronal

NEUROGENIN BOTH ACTIVATES, AND IS INHIBITED BY, THE LATERAL INHIBITION MACHINERY

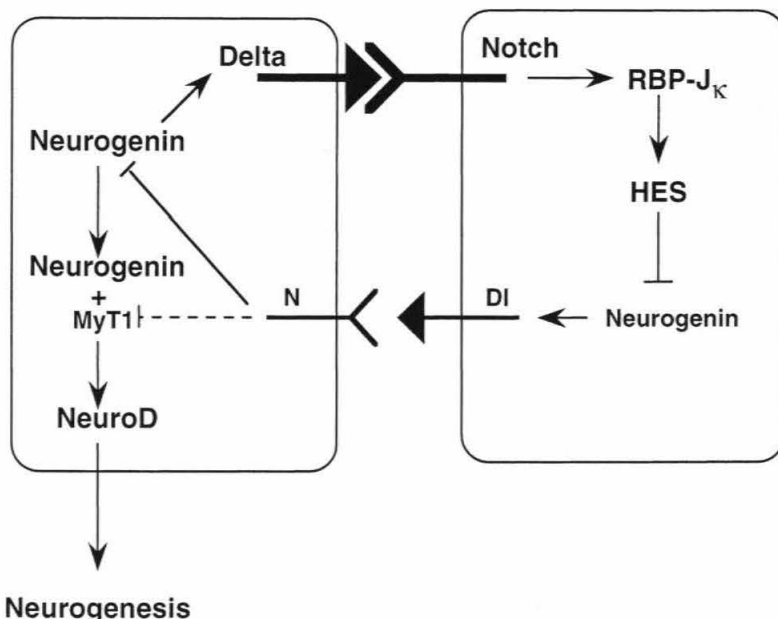


Figure 8. Role of neurogenin in neuronal determination as deduced from ectopic expression experiments in *Xenopus*. Neurogenin positively regulates expression of Delta and is in turn negatively regulated (at both transcriptional and posttranscriptional levels) by signaling through Notch. Neurogenin also activates expression of the zinc finger protein X-MyT1, which appears to collaborate with *ngn* to promote induction of XNeuroD and neuronal differentiation in a manner resistant to inhibition by Notch signaling (Bellefroid et al. 1996). Notch signaling likely involves the mammalian homolog of *Drosophila* Suppressor of Hairless, RBP-J κ , (Artavanis-Tsakonas et al. 1995) as well as homologs of *Drosophila* hairy and Enhancer of Split (HES proteins) (Ishibashi et al. 1995). (Modified from Ma et al. 1996.)

identity, is far from clear. This process is being elucidated by studies of neurogenesis in the peripheral autonomic lineage, which is arguably the best-understood neurogenic lineage in vertebrates.

Emerging evidence is beginning to suggest that neuronal diversity within the autonomic and sensory lineages may be generated by related, but distinct, mechanisms. All autonomic progenitors express a common bHLH protein, MASH1, which appears to be induced by members of the BMP2 subfamily secreted by the tissues to which these progenitors migrate. Additional signals may then act on these progenitors in different locations to induce the expression of other transcription factors, which act in conjunction with MASH1 to specify the final phenotypes of the different autonomic neuron subtypes (sympathetic, parasympathetic, and enteric). Although different classes of autonomic neurons develop in very different locations within the body, different classes of sensory neurons are located together in dorsal root ganglia. The finding that distinct but related subtypes of bHLH proteins, the neurogenins, are expressed by different classes of sensory neuron precursors early in development suggests that sensory neuron diversity, in contrast to autonomic neuron diversity, may be pre-specified at or before the time neural crest cells begin their emigration from the neural tube.

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